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DEVELOPMENT OF NEW RICIN A-CHAIN IMMUNOTOXINS WITH POTENT ANTI-TUMOR EFFECTS AGAINST HUMAN HODGKIN CELLS *IN VITRO* AND DISSEMINATED HODGKIN TUMORS IN SCID MICE USING HIGH-AFFINITY MONOCLONAL ANTIBODIES DIRECTED AGAINST THE CD30 ANTIGEN

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The lymphocyte activation marker CD30 has been shown to be an excellent target for the immunotherapy of human Hodgkin's lymphoma. In order to develop new potent immunotoxins (ITs) against CD30, we chemically linked 6 recently described monoclonal antibodies (MAbs) via SMPT to deglycosylated ricin A-chain (dgA). Cross-blocking experiments demonstrated that these MAbs, termed Ki-2 to Ki-7, recognize 3 different clusters on the CD30 antigen: Ki-2, Ki-4, Ki-5 and Ki-7 recognize cluster A; Ki-6 recognizes cluster B; Ki-3 binds to cluster C. Staining of 29 sections of normal human organs revealed no major cross-reactivity of any MAbs tested. Binding to the CD30 antigen on L540Cy Hodgkin cells was assessed by flow cytometry, and demonstrated high affinities for Ki-2, Ki-3 and Ki-4. The concentration giving 50% of the mean fluorescence intensity (MFI_{50}) was 0.58 µg/ml to 0.78 µg/ml. MAbs Ki-5, Ki-6, and Ki-7 bound much more weakly. The staining intensity of the MAbs correlated with the cytotoxicity of the corresponding ITs. Ki-2.dgA, Ki-3.dgA and Ki-4.dgA inhibited the protein synthesis of L540Cy cells by 50% at concentrations (IC_{50}) of 3.5×10^{-10} M to 4.0×10^{-11} M. The most effective IT, Ki-4.dgA, is 5-fold more potent than previously reported CD30 ricin A-chain ITs. Ki-4.dgA was subsequently used for the treatment of disseminated human Hodgkin's lymphoma in a SCID mouse model. The mean survival time (MST) of lymphoma-bearing SCID mice was extended from 42 days in untreated controls to more than 132 days when Ki-4.dgA was applied one day after tumor challenge. Ki-4.dgA is a new potent IT suitable for further evaluation against Hodgkin's lymphoma in man.

The introduction of extended-field radiotherapy and multiagent chemotherapy such as MOPP (mustargen, oncovin, procarbazine, prednisone) or ABVD (adriamycin, bleomycin, vinblastine, dacarbazine) has increased the probability of curing patients with Hodgkin's lymphoma from less than 5% in 1962 to about 70% at present (Henry-Amar and Somers, 1990). However, 30% to 50% of patients at advanced stages relapse and finally die of their disease. High-dose chemotherapy with autologous bone marrow transplantation (ABMT) has been reported to be effective in patients with relapsed disease, but the number of long-term remissions is low (Pfreundschuh *et al.,* 1994). This might be due, at least in part, to residual Hodgkin-Reed/Sternberg (H-RS) cells surviving radio/chemotherapy (Brousset *et al.,* 1994). Patients who are eventually cured face the risk of infertility or second malignancies, which have been reported to occur in up to 11.2% within 15 years after treatment (Henry-Amar, 1992). Thus, new reagents with non-mutagenic potency are needed which can selectively destroy residual malignant cells.

One promising new approach is the use of ITs consisting of a toxin portion linked to or fused with a binding moiety directed against H-RS-associated antigens. Hodgkin's lymphoma is ideally suited for the use of ITs for the following reasons: (i) the lymphocyte activation markers CD25 and CD30 are expressed in high copy numbers on H-RS cells (Stein *et al.,* 1985); (ii) the number of malignant cells in infiltrated tissues that have to be destroyed is small; (iii) Hodgkin's lymphoma are well vascularized, suggesting sufficient access of the IT to most or all target cells; (iv) Hodgkin's disease is very sensitive to conventional therapy, allowing substantial debulking before ITs are used. The major strength of ITs will probably lie in the treatment of minimal residual disease rather than against large tumor masses in end-stage patients, as has been suggested from recent clinical trials (Grossbard *et al.,* 1993*a,b*; Amlot *et al.,* 1993).

We have demonstrated the cytotoxic potential of ITs against H-RS cells by chemically linking the ricin A-chain to MAbs against CD25 and CD30 (Engert *et al.,* 1990; Winkler *et al.,* 1994). The most potent construct, RFT5γ1.dgA (CD25), is currently being used in a clinical phase-I/II trial in patients with relapsed Hodgkin's lymphoma (Engert *et al.,* 1994). New CD30 ITs are needed, since mixtures ("cocktails") consisting of CD25 and CD30 ITs are more effective than either single construct (Engert *et al.,* 1995). In addition, the CD30 ITs reported earlier were generally not as potent as CD25 ITs (Engert *et al.,* 1990). Thus, we constructed new CD30 ITs utilizing a panel of CD30 MAbs (Horn-Lohrens *et al.,* 1995). In the present study we demonstrate that the most potent new CD30 IT, Ki-4.dgA, is at least 5-fold more effective against H-RS cells *in vitro* than previous CD30 ITs, and has potent anti-tumor effects against disseminated human Hodgkin's lymphoma in SCID mice. Ki-4.dgA will soon be used in clinical trials in patients with Hodgkin's lymphoma.

MATERIAL AND METHODS

Materials

Tissue-culture medium RPMI 1640 was supplemented with 10% FCS, 200 units/ml penicillin, 100 µg/ml streptomycin and

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Abbreviations: ABMT, autologous bone-marrow transplantation; ABVD, adriamycin-bleomycin-vinblastine-dacarbazine; APAAP, alkaline-phosphatase/anti-alkaline-phosphatase antibody; BSA, bovine serum albumin; CD, cluster of differentiation; dgA, deglycosylated ricin A-chain; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FITC, fluorescein-isothiocyanate; GAM, goat-anti-mouse; IC_{50}, 50% inhibitory concentration; H-RS, Hodgkin-Reed/Sternberg; IT, immunotoxin; MFI_{50}, concentration giving 50% of maximal mean fluorescence intensity; MAb, monoclonal antibody; MOPP, mustargen-oncovin-procarbazine-prednisone; MST, mean survival time; MTD, maximum tolerated dose; PBS, phosphate-buffered saline; SCID, severe combined immunodeficiency; SMPT, 4-succinimidyl-oxycarbonyl-α-methyl-α-(2-pyridyldithio) toluene.

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Ex2

2 mM glutamine and were purchased from GIBCO-BRL (Gaithersburg, MD). Sepharose G25 (fine grade), Sephacryl S200 HR, and blue Sepharose CL-6B were obtained from Pharmacia (Uppsala, Sweden). Ricin A chain was a generous gift from Dr. P. Thorpe (Cancer Immunobiology Center, Dallas, TX). 4-succinimidyl-oxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT) was purchased from Pierce (Rockford, IL). [^{3}H]-leucine was from Amersham Buchler (Braunschweig, Germany). Falcon tissue-culture flasks were purchased from Becton Dickinson (Oxnard, CA). Biotinamidocaproate-N-hydroxysuccimide was purchased from Sigma (St. Louis, MO) and DMSO from Merck (Darmstadt, Germany). Mouse anti-human MAb Ber-H2 (CD30), mouse anti-human CD13 MAb, biotinylated rabbit anti-mouse MAb E 413, and a streptavidin-biotin-complex labeled with alkaline phosphatase (K 391) were from Dako (Hamburg, Germany). HRS-3 (IgG1) was a kind gift from Dr. M. Pfreundschuh (Homburg, Germany). The mouse MAb OX7 (IgG$_1$), recognizing the mouse Thy 1.1 antigen, was used as non-specific isotype control.

Cells

The cell line L540Cy was maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin ("complete medium"). Cells were cultured in an atmosphere of 5% CO_2 and humidified air. The L540Cy is a mutant of the L540 cell line, and has been shown to have superior take rates in mice as compared with the parental L540. Chinese hamster ovary (CHO) cells grew as adherent cells in complete medium. For the preparation of cell suspensions, the cells were detached by trypsin-EDTA solution (10%). HL60, an erythrocytic-monocytic acute myeloid leukemia cell line, was used as a control not expressing the CD30 antigen.

CD30 antibodies

The CD30 MAbs used in this study were Ki-1 (IgG$_3$), Ki-2 (IgG$_1$), Ki-3 (IgG$_{2b}$), Ki-4 (IgG$_1$), Ki-5 (IgG$_1$), Ki-6 (IgG$_1$), and Ki-7 (IgG$_1$). Ki-2, Ki-4, Ki-5 and Ki-7 have been suggested to bind to the same cluster of epitopes (A) on the CD30 antigen as the MAbs, BerH2 and HRS-3, whereas Ki-6, like Ki-1, binds to cluster B, and Ki-3 binds to cluster C (Horn-Lohrens *et al.*, 1995).

Preparation of ITs

The ITs were prepared as described by Thorpe *et al.* (1988). In brief, MAbs were treated with the heterobifunctional linker SMPT to introduce an average of 1.7 activated disulfide groups per antibody molecule. The derivatized protein was separated from unreacted material by gel chromatography on a Sephadex G-25 column and mixed with freshly reduced deglycosylated ricin A-chain. After 72 hr, residual thiol groups were inactivated with 0.2 mM cysteine. The IT was then purified from unreacted ricin A-chain, cysteine and high-molecular-weight aggregates on a Sephacryl S200HR column. Finally, free antibody was removed by chromatography on a blue Sepharose CL-6B column.

Staining of normal and malignant human tissues

To exclude cross-reactivity with vital human organs, a panel of normal human tissues was stained with the CD30 MAbs by standard immuno-peroxidase or immunoalkaline-phosphatase methods. To confirm the specificity of the MAbs used, tissues of malignant entities were stained, including breast carcinoma, gastrointestinal carcinoma, squamous carcinoma, malignant melanoma, T- and B-cell lymphoma, Ki-1 lymphoma and Hodgkin's lymphoma.

FACS analysis

Cell suspensions (100 μl) containing 5 × 10^6/ml L540Cy cells were incubated for 15 min at 4°C with different concentrations of CD30 MAbs ranging from 5 × 10^{-4} μg/ml to 50 μg/ml, washed 3 times, and subsequently incubated with fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin for another 15 min at 4°C, then washed again. Indirect immunofluorescence was measured on a Becton Dickinson FACScan (fluorescence-activated cell sorter). The percentage of positively stained cells under saturating concentration of MAbs (5–50 μg/ml) as compared with the non-binding control MAb (anti-CD13) was calculated. Binding of the MAbs to L540Cy target cells was assessed by determining the concentration of antibody giving 50% of the maximal fluorescence intensity (MFI$_{50}$).

Cross-blocking experiments

Cross-blocking experiments were performed by comparing the staining intensity of native and biotinylated CD30 MAbs. Biotinylation was carried out using standard methods. Triplicate samples of 100 μl L540Cy cells (5 × 10^6/ml) were mixed with biotinylated antibody (1.0 μg/ml, 100 μl) and a 100-fold excess of various unlabeled antibodies. These mixtures were incubated for 30 min at 4°C and then washed 3 times with PBS. Staining was performed with FITC-labeled streptavidin. Analysis of fluorescence intensity was evaluated on a Becton Dickinson FACScan. Blocking of labeled MAbs was calculated as percentage of fluorescence of unblocked controls.

Cytotoxicity assays

The indirect cytotoxicity assays were performed as described by Till *et al.* (1988). Briefly, triplicates (100 μl) of unconjugated MAb in complete medium were distributed into 96-well plates and diluted to give final concentrations ranging from 1 × 10^{-8} M to 1 × 10^{-13} M. Then 100 μl of the same medium, containing 4 × 10^4 L540Cy Hodgkin cells were added to each well. After incubation for 30 min at 4°C, Fab' goat anti-mouse (GAM)Ig.dgA was added as second layer to give a final concentration of 2 μg/ml, and the plates were incubated for 24 hr at 37°C in humidified air in an atmosphere of 5% CO_2, pulsed with 1 μCi/well [^{3}H]-leucine, and incubated for a further 24 hr. The cells were then harvested onto glass-fiber filters using a Titertek cell harvester and the incorporated [^{3}H]-leucine was measured by liquid scintillation (Beckmann, LS/801, San Ramon, CA).

Thereafter the ITs constructed were tested against L540Cy Hodgkin cells in standard [^{3}H]-leucine-uptake assays. The plates were incubated for 24 hr, pulsed and harvested as described above for the indirect assays. Concentrations at which the [^{3}H]-leucine incorporation was inhibited by 50% relative to untreated control cultures (IC$_{50}$) were calculated.

Animals

The SCID mice were obtained from our own colony and maintained under sterile conditions. No antibiotic prophylaxis was provided. ELISAs were performed regularly to detect whether antibodies had leaked into the sera of the animals.

Anti-tumor experiments in mice

Adult 4- to 6-week-old SCID mice were injected i.v. via the tail vein with 1 × 10^7 L540Cy Hodgkin cells suspended in 600 μl of PBS containing 0.2% BSA. Vital signs were recorded daily, and weight was measured once per week. The SCID mice were killed when developing signs of progressive disease including weight loss, ruffled fur, and inactivity. After gross examination, organs macroscopically infiltrated with L540Cy lymphoma cells were snap-frozen in liquid nitrogen. Cytocentrifuge slides were prepared from bone marrow extracted from tibiae. Selected mouse organs and cytocentrifuge slides were immunostained using the alkaline-phosphatase/anti-alkaline-phosphatase antibody (APAAP) method.

To evaluate the effects of IT treatment on the survival of SCID mice challenged with human Hodgkin-lymphoma cells, animals were randomly divided into groups of 5. One day after

TABLE I – REACTIVITY OF CD30 MAbs WITH NORMAL AND MALIGNANT HUMAN TISSUES

	Ki-2	Ki-3	Ki-4	Ki-5	Ki-6	Ki-7
Neural tissue	–	–	–	–	–	–
Cardiovascular system	–	–	–	–	–	–
Bronchopulmonary system	–	–	–	–	–	–
Orolaryngeal tract	–	–	–	–	–	–
Hepatobiliary system	–	–	–	–	–	–
Gastrointestinal tract	–	–	–	–	–	–
Pancreas	(+/–)	(+/–)	(+/–)	(+/–)	(+/–)	(+/–)
Salivary gland	–	–	–	–	–	–
Cartilage/synovia/bone	–	–	–	–	–	–
Muscles	–	–	–	–	–	–
Bone marrow	–	–	–	–	–	–
Blood cells	–	–	–	–	–	–
Lymph node/spleen/thymus/tonsils	–	–	–	–	–	–
Mammary glands	–	–	–	–	–	–
Endocrine glands	–	–	–	–	–	–
Kidney	–	–	–	–	–	–
Urinary tract	–	–	–	–	–	–
Testis/epididymis	(+/–)	(+/–)	(+/–)	(+/–)	(+/–)	(+/–)
Prostate/seminal vesicle	–	–	–	–	–	–
Uterus/ovary/placenta	–	–	–	–	–	–
Breast carcinoma	–	–	–	–	–	–
Gastrointestinal carcinoma	–	–	–	–	–	–
Squamous carcinoma	–	–	–	–	–	–
Malignant melanoma	–	–	–	–	–	–
T- and B-cell lymphoma	–	–	–	–	–	–
Ki-1 lymphoma (large-cell, anaplastic)	+	+	n.e.	n.e.	+	+
Hodgkin's disease	+	+	+	+	+	+

–, no staining; +/–, weak or uncertain staining; +, positive staining; n.e., not examined.

inoculation of L540Cy Hodgkin cells, mice received a single i.p. injection of IT (Ki-4.dgA), of unconjugated MAb (Ki-4) or of PBS in a volume of 400 µl PBS containing 0.2% BSA. The dose of IT (48 µg of total protein corresponding to 8 µg of ricin A-chain) represented the maximum tolerated dose (MTD) in SCID mice determined in a dose-escalation study of groups of 3 mice. The doses of unconjugated MAb matched those in the immunotoxin (40 µg).

RESULTS

Staining of normal and malignant human tissues

In all, 29 normal tissues and 7 malignant tumors, including Hodgkin's lymphoma, were stained with the MAbs Ki-2 to Ki-7 (Table I). All MAbs had a similar staining pattern on normal tissues, showing no cross-reactivity with vital human organs. There were only very weak or uncertain reactions with some activated cells in the pancreas, testis and epididymis. No staining apart from Hodgkin's and non-Hodgkin's lymphoma tissue of either of the 6 MAbs tested was observed with solid human tumors. The human Hodgkin cell lines L428 and L540 were consistently positive for all MAbs (data not shown).

Binding of CD30 MAbs on L540Cy cells

The binding capacity of the new CD30 MAbs was estimated from the MFI_{50} as measured by FACS analysis, using the method of Shen *et al.* (1988). This method reveals relative differences between MAbs in terms of binding capacity, but does not represent a true measure of affinity, because of washing cells before analysis. The results are listed in Table II. The binding of the MAbs Ki-1, Ki-2, Ki-3, Ki-4 and Ki-7 was 77- to 180-fold stronger than that of Ki-5 and Ki-6. Ki-7 had much weaker mean fluorescence intensity than the other MAbs investigated (data not shown). HRS-3 and Ber-H2 showed a binding capacity 2- to 8-fold less than that of MAbs Ki-1 to Ki-4.

Cross-blocking of CD30 monoclonal antibodies

These experiments were performed to determine the epitopes on the CD30 antigen recognized by MAbs Ki-1 to Ki-7. The

TABLE II – BINDING CAPACITY OF CD30 MAbs ON L540CY HODGKIN CELLS AS COMPARED BY FACS ANALYSIS

Antibody	MFI_{50}[1]
Ki-1	0.40 ± 0.17
Ki-2	0.66 ± 0.25
Ki-3	0.58 ± 0.31
Ki-4	0.78 ± 0.20
Ki-5	72.5 ± 10.60
Ki-6	60.0 ± 20.0
Ki-7	0.40 ± 0.46
Ber-H2	3.0 ± 0.46
HRS-3	1.5 ± 0.20

[1]µg/ml ± standard deviation of triplicate determinations. Hodgkin-derived L540Cy cells were incubated with different concentrations of the CD30 MAbs and subsequently incubated with fluorescein-labeled goat anti-mouse immunoglobulin. Indirect fluorescence intensity was measured on a FACS fluorometer. Percentages of positive cells compared with a non-binding control MAb (CD13) were calculated.

results are summarized in Table III. Ki-1 and Ki-6 blocked each other's binding to L540Cy cells, but were not blocked by Ki-2, Ki-3, Ki-4, Ki-5 or Ki-7. Ki-6 was slightly more effective at blocking Ki-1 than *vice-versa*. Ki-2 and Ki-5 cross-blocked each other's binding and were both blocked by Ki-4, which was blocked by Ki-2 and Ki-5, but only to a limited extent. Ki-3 was blocked only by itself, and did not block any CD30 MAb tested in this study. Ki-7 could not compete with any MAb tested, and was blocked only partially by itself. On the other hand, none of MAbs Ki-1 to Ki-6 blocked the binding of Ki-7. Thus it appears, as suggested by Horn-Lohrens *et al.* (1995), that at least 3 different clusters exist on the CD30 antigen: cluster A is recognized by MAbs Ki-2, Ki-4, and Ki-5; Ki-1 and Ki-6 bind to cluster B; and Ki-3 recognizes cluster C. In contrast to Horn-Lohrens *et al.* (1995), we could not determine an affinity of Ki-7 to cluster A. This finding might be explained by the very low affinity of Ki-7 to the CD30 antigen and the lower sensitivity of the non-radioactive labeling method used in our study.

TABLE III – CROSS-BLOCKING EXPERIMENTS WITH CD30 MAbs[1]

Cluster	Inhibitors	Biotinylated MAbs						
		Ki-1	Ki-2	Ki-3	Ki-4	Ki-5	Ki-6	Ki-7
	—[2]	1	1	1	1	1	1	1
A	Ki-2	1.03 ± 0.07	0.11 ± 0.01	1.19 ± 0.02	0.61 ± 0.01	0.13 ± 0.00	0.95 ± 0.04	0.99 ± 0.03
	Ki-4	1.44 ± 0.06	0.41 ± 0.01	1.09 ± 0.01	0.16 ± 0.01	0.32 ± 0.03	1.00 ± 0.03	1.11 ± 0.09
	Ki-5	1.32 ± 0.08	0.20 ± 0.01	1.12 ± 0.09	0.57 ± 0.05	0.24 ± 0.02	1.13 ± 0.04	0.93 ± 0.05
	Ki-7	1.07 ± 0.07	1.09 ± 0.02	1.14 ± 0.03	1.06 ± 0.01	0.95 ± 0.12	1.17 ± 0.06	0.63 ± 0.01
B	Ki-1	0.19 ± 0.00	0.72 ± 0.05	0.85 ± 0.07	0.95 ± 0.07	0.71 ± 0.14	0.41 ± 0.00	0.95 ± 0.02
	Ki-6	0.14 ± 0.00	1.00 ± 0.02	1.04 ± 0.03	1.07 ± 0.02	0.85 ± 0.11	0.13 ± 0.00	0.94 ± 0.02
C	Ki-3	0.87 ± 0.07	1.03 ± 0.02	0.16 ± 0.00	0.91 ± 0.03	0.85 ± 0.01	1.06 ± 0.03	1.03 ± 0.02

[1]Cross-blocking of CD30 MAbs. L540Cy cells were mixed with biotinylated CD30 MAbs (1 μg/ml) and subsequently with a 100-fold excess of unlabeled CD30 MAbs. Given values are means of at least 3 experiments ± standard deviation.–[2]Without inhibitor.

TABLE IV – TOXICITY OF NEW CD30 RICIN-A-CHAIN IMMUNOTOXINS AGAINST L540CY CELLS

Reagent	IC_{50}[1]
Ki-1.dgA	$1.0 \pm 0.5 \times 10^{-8}$ M
Ki-2.dgA	$3.5 \pm 0.5 \times 10^{-10}$ M
Ki-3.dgA	$1.3 \pm 0.6 \times 10^{-10}$ M
Ki-4.dgA	$4 \pm 0.1 \times 10^{-11}$ M
Ki-5.dgA	$1.0 \pm 0.5 \times 10^{-8}$ M
Ki-6.dgA	$1.5 \pm 0.4 \times 10^{-9}$ M
Ki-7.dgA	$>1.0 \times 10^{-6}$ M
HRS-3.dgA	$2 \pm 0.5 \times 10^{-10}$ M
BER-H2.dgA	$3 \pm 1.5 \times 10^{-10}$ M
Ricin	$6.0 \pm 2.0 \times 10^{-12}$ M
Ricin A	$8.0 \pm 3.2 \times 10^{-7}$ M
OX7.dgA	$>1 \times 10^{-6}$ M

[1]Concentration giving 50% inhibition of protein synthesis of L540Cy Hodgkin cells. Protein-synthesis inhibition was measured in a [^{3}H]-leucine-incorporation assay. Values are means of triplicate determinations ± standard deviation.

Cytotoxicity of new CD30 ricin A-chain ITs against Hodgkin cells

The CD30 MAbs were first screened for their effectiveness in indirect assays against L540Cy Hodgkin cells. Ki-3 and Ki-4 were very potent, with IC_{50} values of 8×10^{-11} M and 6×10^{-11} M. The remaining MAbs Ki-2, Ki-5 and Ki-6 were moderately effective, with IC_{50} values between 1×10^{-10} M and 5×10^{-9}, and Ki-7 was ineffective, with an IC_{50} value of $> 10^{-6}$ M.

Subsequently the MAbs were coupled via SMPT to deglycosylated ricin A-chain. The cytotoxicity was then evaluated in standard [^{3}H]-leucine-uptake assays. The results of a series of direct cytotoxicity experiments are summarized in Table IV. Figure 1 shows a representative experiment using the ITs Ki-2.dgA, Ki-3.dgA, Ki-4.dgA and HRS-3.dgA. There was a clear correlation of staining intensity/affinity of the parental MAb and cytotoxic potency of the corresponding IT. All ITs derived from high-affinity CD30 MAbs were potently toxic against Hodgkin cells *in vitro*. The most powerful new CD30 IT, Ki-4.dgA, inhibiting the protein synthesis of L540Cy cells at IC_{50} of 4×10^{-11} M, was only 8-fold less effective than unmodified ricin. Ki-4.dgA was 3.3-, 8.8- and 37.4-fold more effective than the next potent new CD30 ITs Ki-3.dgA, Ki-2.dgA and Ki-6.dgA respectively. In addition, Ki-4.dgA was 5- and 7.5-fold more effective in the same experimental conditions than the CD30 ITs HRS-3.dgA and BerH2.dgA described earlier (Engert *et al.*, 1990). The ITs derived from low-affinity MAbs Ki-5 and Ki-7 were relatively ineffective, as reported for another low-affinity CD30 IT, Ki-1.dgA (Engert *et al.*, 1990).

The cytotoxic effect of all ITs tested was specific, since neither the native antibodies nor an irrelevant control IT, OX7.dgA, were toxic at 10^{-6} M. The AML-derived cell line HL60, which is negative for CD30, was not affected by the CD30 ITs (data not shown).

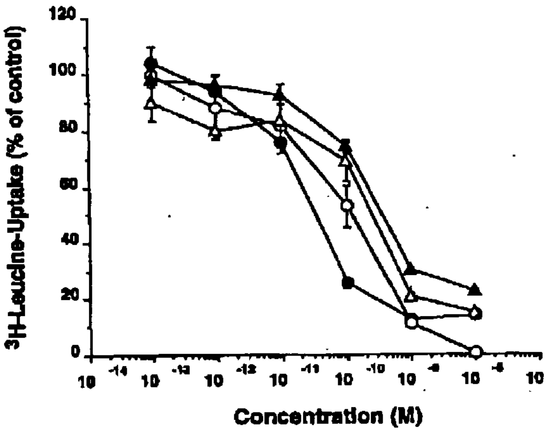

FIGURE 1 – Cytotoxicity of CD 30 immunotoxins to L540Cy cells *in vitro*. The cells were incubated for 48 hr with Ki-2.dgA (▲), Ki-3.dgA (○), Ki-4.dgA (●) or HRS-3.dgA (△). Points, geometric means of triplicate measurements of ^{3}H-leucine incorporation by the cells during the last 24-hr period of culture expressed as a percentage of the incorporation of untreated cultures. Bars, one standard deviation of triplicate determinations.

Effect of Ki-4.dgA treatment on the survival of SCID mice challenged with L540Cy Hodgkin cells

Groups of 5 mice were treated with Ki-4.dgA, Ki-4 MAb or PBS 1 day after tumor challenge with L540Cy Hodgkin cells. As illustrated in Figure 2, the MST of the PBS-treated control group was 41.2 (±3.4) days. All mice in this control group had massive tumor burden, with involvement of lymph nodes (90%), liver (50%) and other extranodal sites (20%), as judged macroscopically, and in some cases by histologic examination (Figs. 3, 4). The results of treatment with Ki-4 MAb (40 μg) alone are preliminary, since only 5 mice in all were evaluable, but treatment extended the MST by 9 days without inducing complete remission. L540Cy tumor involvement of organs showed the same distribution pattern as the PBS-treated control group. In contrast, 5 of 10 mice treated with 8 μg (in terms of A-chain) of Ki-4.dgA one day after the inoculation of L540Cy cells were in continuous complete remission more than 200 days after treatment. This result is statistically significant ($p < 0.01$) as compared with the PBS-treated control group. Three animals died for unknown reasons 43, 48 and 61 days after tumor challenge, and showed no detectable L540Cy cells upon examination. The 2 other mice treated with Ki-4.dgA died 70 and 98 days after treatment, from progressive lymphoma with involvement of lymph nodes, liver, spleen and kidney, as demonstrated by APAAP immunostaining.

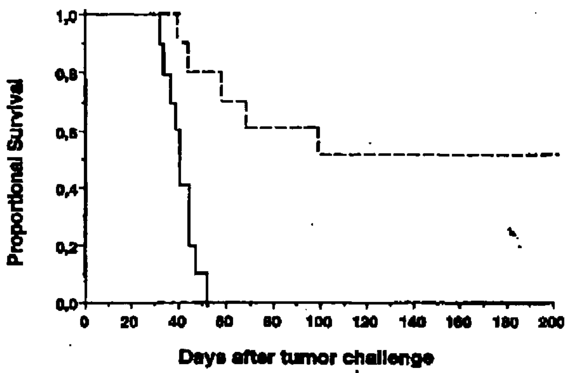

FIGURE 2 – Anti-tumor effect of the immunotoxin Ki-4.dgA on disseminated L540Cy Hodgkin tumors in SCID mice as measured by survival. Groups of 10 animals were treated 1 day after tumor-cell inoculation with PBS (—) or Ki-4.dgA (——).

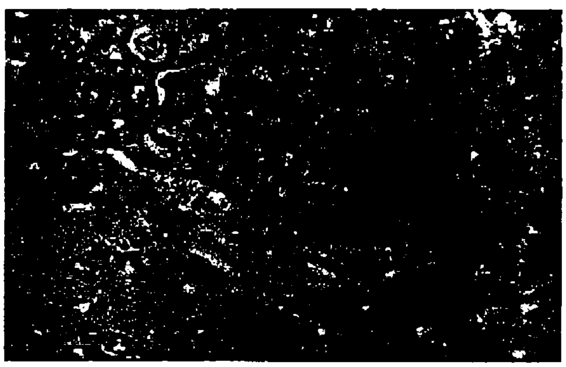

FIGURE 3 – Immunohistopathology (staining by Ber-H2) of Hodgkin-derived L540Cy cells forming a solid tumor in SCID mice infiltrating the liver. At the bottom, normal liver parenchym; 4-μm paraffin section. Scale bar, 3.2 μm.

DISCUSSION

We evaluated 6 new CD30 MAbs, termed Ki-2 to Ki-7, for their potential use as ricin A-chain ITs against human Hodgkin's lymphoma. The major findings to emerge from this study are: (i) all CD30 MAbs evaluated showed high specificity, and restricted binding to normal human tissues, making their use as immunoreagents possible; (ii) three of the new CD30 ITs constructed, including Ki-4.dgA, Ki-3.dgA and Ki-2.dgA, were highly potent against human Hodgkin cells *in vitro*, inhibiting the protein synthesis of L540Cy cells by 50% at concentrations of 4×10^{-11} M, 1.3×10^{-10} M and 3.5×10^{-10} M respectively. Ki-4.dgA was 5-fold more effective than the most potent CD30 ricin A-chain IT (HRS-3.dgA) described earlier (Engert *et al.*, 1990); (iii) treatment of SCID mice with a single i.p. injection of Ki-4.dgA 1 day after challenge with L540Cy cells extended mean survival 3-fold, as compared with controls treated with PBS or Ki-4 MAb alone.

The CD30 antigen has increasingly attracted attention, both for diagnostic and for therapeutic purposes. It has recently been cloned, and has been shown to be a 120-kDa membrane protein belonging to the nerve-growth-factor-receptor (NGFR) superfamily (Dürkop *et al.*, 1992). In addition, the sequence of the murine and human ligand for CD30 (CD30L) has been identified as type-II membrane protein, which can stimulate the proliferation of CD3-activated peripheral T cells and Hodgkin-derived lymphoma cell lines L540 and HDLM-2 (Gruss *et al.*, 1994). In contrast, CD30L induces cell death in several other CD30-positive lymphoma cell lines (Gruss *et al.*, 1994).

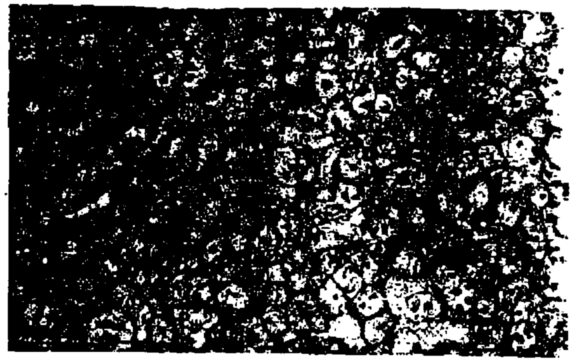

FIGURE 4 – Bone-marrow infiltration of a femur by L540Cy cells in SCID mice, showing mitotic figures and polynuclear appearance. Immunohistologic staining by Ber-H2; 4-μm paraffin section. Scale bar, 3.2 μm.

In addition to Hodgkin's lymphoma, CD30 is found on anaplastic large-cell lymphomas (Stein *et al.*, 1985), T-cell lymphomas (Stein *et al.*, 1985), and occasionally on some carcinoma cells (Pallesen and Hamilton-Dutoit, 1988). Physiologically, macrophages at a late stage of their differentiation, as well as activated T-cells, express CD30 (Falini *et al.*, 1995). Because of the high number of CD30 on malignant H-RS cells and the low expression on normal tissues, this antigen is suitable for immunotherapy. After the reports by our group on the anti-tumor effects of CD30, constructed by chemically linking the antibody moiety to the A-chain of ricin (Engert *et al.*, 1990), others have described anti-tumor effects against H-RS cells using CD30-based ITs constructed with saporin (Falini *et al.*, 1992*a*) or cytostatic pro-drugs (Sahin *et al.*, 1990). Labeling experiments in patients with Hodgkin's lymphoma, using the CD30 MAb BerH2, underscored the suitability of CD30 for immunotherapy: biopsies taken from involved tissue in patients with refractory Hodgkin's lymphoma 24 to 48 hr after the administration of a mixture of 0.5 to 1.0 μg of ^{131}I-labeled BerH2 and 14 to 39 μg unlabeled Ber-H2 demonstrated that virtually all H-RS cells in different organs were specifically and strongly stained (Falini *et al.*, 1992*b*).

Falini *et al.* (1992*a*) described impressive tumor reduction in 3 of 4 patients with refractory Hodgkin's lymphoma after treatment with the CD30 MAb Ber-H2 chemically linked to saporin-6. Duration of response was generally short. Toxicity observed included reversible WHO-grade IV liver toxicity, which proved dose-limiting. By far the largest clinical experience with ITs has been gained with ricin-based constructs (reviewed in Vitetta *et al.*, 1993). More than 200 patients with resistant non-Hodgkin's lymphoma (NHL) have been enrolled in phase-I/II protocols evaluating different schedules, such as bolus administration or continuous infusion (reviewed in Vitetta *et al.*, 1993). The best results were observed in lymphoma patients with less than 100 g of tumour tissue (Amlot *et al.*, 1993). Impressive clinical results have been observed using ITs in which ricin was blocked sterically: one of these constructs, B4-blocked-ricin (CD19), has demonstrated

anti-tumor effects in phase I/II in patients with NHL (Grossbard *et al.*, 1993*b*) and is under investigation in a phase-III trial.

Based on our earlier pre-clinical investigations, we are currently using a chemically linked CD25 ricin A-chain IT (RFT5γ1.dgA) in a phase-I/II trial in patients with refractory Hodgkin's lymphoma. This IT has been shown to be one of the most potent ITs known (Engert *et al.*, 1991). The best new CD30 IT described in the present paper, Ki-4.dgA, seems to match RFT5γ1.dgA in terms of anti-tumor efficacy, though direct comparisons have not been performed. Ki-4.dgA was at least 5-fold more effective than other CD30 ricin A-chain ITs, such as HRS3.dgA (Engert *et al.*, 1990). This might, at least in part, be due to the higher affinity of Ki-4. A clear correlation of staining intensity/affinity of the parental MAb or Fab' fragment and cytotoxic potency of the corresponding IT has been demonstrated for the CD30 antigen (Engert *et al.*, 1990) and for the CD22 antigen (Shen *et al.*, 1988), using Scatchard and FACS analysis. Another possible reason for the superior cytotoxicity of Ki-4.dgA may be the fact that Ki-4 inhibits the release of sCD30 from H-RS cells (Horn-Lohrens *et al.*, 1995). This could be an important argument for the clinical use of Ki-4.dgA, since sCD30 is being phased out, probably as a result of disease activity and infiltration of tumor mass into the sera of Hodgkin's-lymphoma patients (Berenbeck *et al.*, 1989).

We intend to use the new CD30 IT Ki-4.dgA, together with the CD25 IT RFT5γ1.dgA, in patients with Hodgkin's disease, since the concomitant use of 2 or more ITs ("cocktails") against different antigens on H-RS cells has been demonstrated to enhance the anti-tumor effect (Engert *et al.*, 1995). Others have reported IT cocktails to be superior to single ITs in NHL (Ghetie *et al.*, 1992), T-cell lymphoma (Katz *et al.*, 1987), and non-T-cell leukemias (Hara *et al.*, 1988). In addition, preliminary results from our ongoing clinical trial with RFT5γ1.dgA suggest weaker CD25 expression in some heavily pre-treated HD-patients. Interestingly, these H-RS cells still seem to express CD30 strongly, suggesting that CD25/CD30 IT cocktails would be effective.

In conclusion, the new potent CD30 IT, Ki-4.dgA, warrants further evaluation for the treatment of Hodgkin's lymphoma in humans.

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